

## Transforming growth factor type $\beta$ : Rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*

ANITA B. ROBERTS\*, MICHAEL B. SPORN\*, RICHARD K. ASSOIAN\*, JOSEPH M. SMITH\*, NANETTE S. ROCHE\*, LALAGE M. WAKEFIELD\*, URSULA I. HEINE\*, LANCE A. LIOTTA\*, VINCENT FALANGA†, JOHN H. KEHRL‡, AND ANTHONY S. FAUCI‡

\*National Cancer Institute, Bethesda, MD 20892; †University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; and ‡National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892

Communicated by Roy Hertz, February 24, 1986

**ABSTRACT** Transforming growth factor type  $\beta$  (TGF- $\beta$ ), when injected subcutaneously in newborn mice, causes formation of granulation tissue (induction of angiogenesis and activation of fibroblasts to produce collagen) at the site of injection. These effects occur within 2–3 days at dose levels of  $<1 \mu\text{g}$ . Parallel *in vitro* studies show that TGF- $\beta$  causes marked increase of either proline or leucine incorporation into collagen in either an NRK rat fibroblast cell line or early passage human dermal fibroblasts. Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) do not cause these same *in vivo* and *in vitro* effects; in both rat and human fibroblast cultures, EGF antagonizes the effects of TGF- $\beta$  on collagen formation. We have obtained further data to support a role for TGF- $\beta$  as an intrinsic mediator of collagen formation: conditioned media obtained from activated human tonsillar T lymphocytes contain greatly elevated levels of TGF- $\beta$  compared to media obtained from unactivated lymphocytes. These activated media markedly stimulate proline incorporation into collagen in NRK cells; this effect is blocked by a specific antibody to TGF- $\beta$ . The data are all compatible with the hypothesis that TGF- $\beta$  is an important mediator of tissue repair.

Transforming growth factor type  $\beta$  (TGF- $\beta$ ) was originally identified in neoplastic cells (1–4) and then found in a wide variety of non-neoplastic tissues (1–5); the first total purification of this 25-kDa peptide was from three non-neoplastic sources—namely, human blood platelets, human placenta, and bovine kidney (6–8). Recently, the human gene for TGF- $\beta$  has been cloned (9), and mRNA transcripts have been found in both neoplastic and non-neoplastic cells. There is a marked induction of TGF- $\beta$  mRNA when peripheral blood lymphocytes are activated (9). The presence of TGF- $\beta$  in cells or cell fragments of hematopoietic origin suggests that it may play some intrinsic role in inflammation and tissue repair. It had been shown previously that extrinsic TGF- $\beta$  could stimulate the formation of connective tissue and collagen within wire-mesh wound-healing chambers implanted in rats (10, 11). We now report that subcutaneous injection of  $<1 \mu\text{g}$  of TGF- $\beta$  in newborn mice induces angiogenesis and causes rapid activation of fibroblasts to produce collagen; the new tissue formed after injection of TGF- $\beta$  is essentially granulation tissue, resembling that found during physiological wound repair. Furthermore, we show that TGF- $\beta$  *in vitro* has marked stimulatory effects on the formation of collagen by a variety of rodent and human fibroblasts and that these effects are different from those of two other peptide growth factors that have been implicated in tissue repair—namely, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF).

Activated lymphocytes and macrophages have long been implicated in the production of growth factors that stimulate fibroblast proliferation and collagen synthesis, although the chemical nature of many of the specific peptide growth factors that are involved has not been well-defined (12–16). Here, we report that T lymphocytes secrete greatly elevated levels of TGF- $\beta$  into their culture medium when they are activated *in vitro* (17) and that the conditioned media from such activated lymphocytes can stimulate proline incorporation into collagen in rodent fibroblasts. Moreover, we show that specific antibodies to TGF- $\beta$  will partially abolish this stimulatory effect. The present data thus indicate that TGF- $\beta$  is a functionally significant component of the set of lymphokines produced by activated lymphocytes.

### MATERIALS AND METHODS

**Growth Factors.** TGF- $\beta$ , homogeneous by analysis on sodium dodecyl sulfate/polyacrylamide gels, was purified from human platelets (6). EGF was purified from male mouse salivary glands (1). Pure human PDGF was the generous gift of Russell Ross and Elaine Raines and contained  $<0.1\%$  TGF- $\beta$  by radioreceptor assay (18).

***In Vivo* Studies.** Carrier-free sterile TGF- $\beta$  (40  $\mu\text{g}$ ) was dissolved in 200  $\mu\text{l}$  of 2 mM HCl to which 600  $\mu\text{l}$  of phosphate-buffered saline (PBS) and 200  $\mu\text{l}$  of PBS containing NaCl at 9 mg/ml was added. Swiss mice (1 day old) were injected subcutaneously each day in the nape of the neck with a 27-gauge needle, with 20  $\mu\text{l}$  of TGF- $\beta$  or with saline control, and tissue samples were obtained as described in *Results*. Sterile murine EGF was also injected in the same vehicle in the same manner. At the end of each experiment, the firm nodular tissue at the site of injection of TGF- $\beta$  (or the corresponding area at the site of injection of saline or EGF in the control animals) was removed and fixed in either neutral formalin or glutaraldehyde. Paraffin sections of the samples fixed in formalin were then stained with either hematoxylin and eosin or with Masson trichrome (for collagen). Samples of tissue fixed in glutaraldehyde were examined by electron microscopy.

**Cell Culture.** NRK cells, clone 49F, were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 10% calf serum (GIBCO) supplemented with penicillin (50 units/ml) and streptomycin (50  $\mu\text{g}/\text{ml}$ ) in humidified 5%  $\text{CO}_2/95\%$  air at 37°C. Normal human dermal fibroblasts, cultured from biopsies taken from dorsal forearm skin of donors, were grown under identical conditions except that fetal bovine serum was used.

**Assays for Collagen Formation *in Vitro*.** NRK 49F cells were seeded in 1 ml of DMEM/10% calf serum, in 24-well multi-dishes ( $1 \times 10^5$  cells per 16-mm well). After the cells

reached confluence, the medium was changed to minimal essential medium (MEM) (containing glutamine, 20 mM Hepes buffer, and 2% plasma-derived serum), and growth factors were added in 20  $\mu$ l of 4 mM HCl containing bovine serum albumin at 1 mg/ml. After 16 hr, the medium was changed to 300  $\mu$ l per well (containing 0.25 mM ascorbate and other supplements as described above, but without plasma); growth factors were re-added. After 15 min, 6  $\mu$ Ci of L-[2,3-<sup>3</sup>H]proline (29.1 Ci/mmol; 1 Ci = 37 GBq) was added and the incubation continued for 3 hr at 37°C. Collagen was determined on the pooled medium from triplicate wells using bacterial collagenase (Advance Biofactures, Lynbrook, NY) by a modification (15) of the method of Peterkofsky (19).

**Measurement of Amino Acid Uptake.** NRK cells ( $3 \times 10^5$ ) were seeded in 3 ml of medium in six-well (35 mm) multi-dishes and treated with growth factors as described above for collagen determinations. For the uptake experiments, 1  $\mu$ Ci of [1-<sup>14</sup>C]methylaminoisobutyric acid (48.4 mCi/mmol; New England Nuclear) was added to each well (1 ml of MEM supplemented as for collagen determinations) and incubated for 6 min at 37°C, followed by four rapid washes with PBS at 0°C. The washed cells were dissolved in 0.5 M NaOH/1% NaDodSO<sub>4</sub>, and the radioactivity was determined in a scintillation counter (20). Nonspecific uptake, measured in the presence of 10 mM L-proline, was subtracted from all values. Collagen determinations were made on duplicate wells to facilitate direct comparison of the two measurements. All results were normalized to cell number.

**Antibodies to TGF- $\beta$ .** Human platelet TGF- $\beta$  of >98% purity (6) was coupled to keyhole limpet hemocyanin (KLH). Rabbits were immunized with three doses of KLH-TGF- $\beta$  (100  $\mu$ g of TGF- $\beta$  per dose per rabbit) in Freund's adjuvant at 3-week intervals. The IgG fraction was purified from the resulting antiserum by affinity chromatography on protein A-Sepharose (21). Anti-TGF- $\beta$  antibodies prepared in this way inhibited the binding of TGF- $\beta$  to its specific receptors on NRK cells and also suppressed the growth of NRK cells in soft agar in response to exogenous TGF- $\beta$  (L.M.W., unpublished observations). The IgG fraction of normal rabbit preimmune serum prepared in the same way served as a control. Both IgG preparations were extensively dialyzed against PBS and contained 4–5 mg of protein per ml. For determination of the effect of antibodies on collagen formation, growth factor solutions in MEM plus 2% plasma-derived serum were preincubated overnight at 4°C in the presence of immune or preimmune IgG at 40  $\mu$ g/ml, then added directly to the cells and incubated for an additional 16 hr at 37°C prior to determination of proline incorporation.

**Determination of TGF- $\beta$  in Media Conditioned by Lymphocytes.** Tonsillar T lymphocytes were prepared and cultured as described (17). Serum-free HB-102 medium, conditioned by  $10^6$  cells per ml, was dialyzed against 1 M acetic acid, lyophilized, and reconstituted (20- to 50-fold more concentrated) in 4 mM HCl containing bovine serum albumin (1 mg/ml). Concentrations of TGF- $\beta$  were determined both by a competitive radioreceptor assay measuring binding of <sup>125</sup>I-labeled TGF- $\beta$  to A549 human lung carcinoma cells (18) and by comparison of dilution curves of colonies of NRK cells (assayed in the presence of 0.8 nM EGF) in soft agar induced either by standard human platelet TGF- $\beta$  or by the lymphocyte conditioned medium (1).

## RESULTS

**In Vivo Studies.** We have performed six sets of *in vivo* experiments, involving a total of 210 newborn mice, in which either TGF- $\beta$ , EGF, or a saline control vehicle has been injected once daily, subcutaneously, in the nape of the neck. The routine dosages of TGF- $\beta$  and EGF were each 800 ng, in 20  $\mu$ l of saline. Within 48 hr, a firm lump could easily be

palpated at the injection site in mice dosed with TGF- $\beta$ . Such a lump was not seen in mice dosed with either EGF or saline; although EGF did not cause any direct response at the site of injection in these experiments, it was active *in vivo*, as measured by its ability to enhance eyelid opening (22, 23). At the end of each experiment, the firm nodular tissue at the site of injection was removed and fixed in either neutral formalin or glutaraldehyde. Paraffin sections were then stained with either hematoxylin and eosin or Masson trichrome (for collagen). We have examined a total of 64 specimens from mice treated with TGF- $\beta$ ; in every case, there has been a striking induction of the formation of new granulation tissue, as seen in Fig. 1. Microscopic examination of the specimens from the mice treated with EGF showed no significant formation of any granulation tissue; these samples were virtually identical to the saline controls. As shown in Fig. 1, there is both a fibrotic (Fig. 1 B and C) and an angiogenic (Fig. 1 C and D) response to the injection of TGF- $\beta$ , consistent with the characteristic dual response of proliferation of fibroblasts and small blood vessels that occurs during physiological wound healing and tissue repair (24). Masson trichrome staining showed that TGF- $\beta$  caused formation of new collagen as soon as 48 hr after administration (Fig. 1B); this was confirmed by electron microscopy, which showed that TGF- $\beta$  (but neither EGF nor saline) treatment resulted in activation of rough endoplasmic reticulum in fibroblasts and production of extracellular collagen fibrils. Full details of these morphological observations will be published elsewhere.

The response to TGF- $\beta$  is both dose and time dependent. Daily injection of mice with 400 and 200 ng of TGF- $\beta$  resulted in lesser, but detectable, responses when tissues were examined on the third day. Furthermore, the response to TGF- $\beta$  is reversible; when mice were injected daily for 8 consecutive days with 800 ng of TGF- $\beta$ , a large area of granulation tissue was found, whereas injection of TGF- $\beta$  for 3 days, followed by 5 days of rest, resulted in almost total disappearance of the granulation response. Neither EGF (800 ng daily) nor saline treatment for 8 days caused any significant granulation response.

**In Vitro Studies.** To obtain further information about the mechanism of action of TGF- $\beta$  in promoting the fibrotic response, we have performed an extensive series of experiments on cultured human and rat fibroblasts. As seen in Table 1, early-passage human dermal fibroblasts respond to TGF- $\beta$  treatment with a selective stimulation of proline incorporation into collagen, as compared to noncollagen protein. EGF itself does not stimulate incorporation of proline into collagen, and, when added together with TGF- $\beta$ , antagonizes the effects of TGF- $\beta$ . Other cell types, including bovine aorta smooth muscle cells (data not shown), human gingival fibroblasts (J. Sodek and J. Wrana, personal communication), as well as established rodent cell lines (NRK and NIH 3T3), have also been shown to have similar responses to TGF- $\beta$ .

These effects were then studied in greater detail in the NRK cell line. Fig. 2A shows that TGF- $\beta$  (but not EGF) treatment resulted in a dose-dependent stimulation of incorporation of [<sup>3</sup>H]proline into secreted collagen of NRK rat fibroblasts (up to a 7-fold increase); Fig. 2B shows that both TGF- $\beta$  and, to a lesser extent, EGF increased incorporation of proline into noncollagen proteins. The net result, as seen in Fig. 2C, is for TGF- $\beta$  to increase the relative percentage of collagen (with respect to total protein) secreted by the NRK cell.

The ability of EGF to antagonize the TGF- $\beta$ -dependent stimulation of proline incorporation into collagen is also dose dependent; 40 and 200 pM EGF reduce the extent of stimulation resulting from treatment with 50 pM TGF- $\beta$  by 20% and 110%, respectively, and the stimulatory effect of 5 pM TGF- $\beta$  is completely blocked by 40 pM EGF. Pulse-

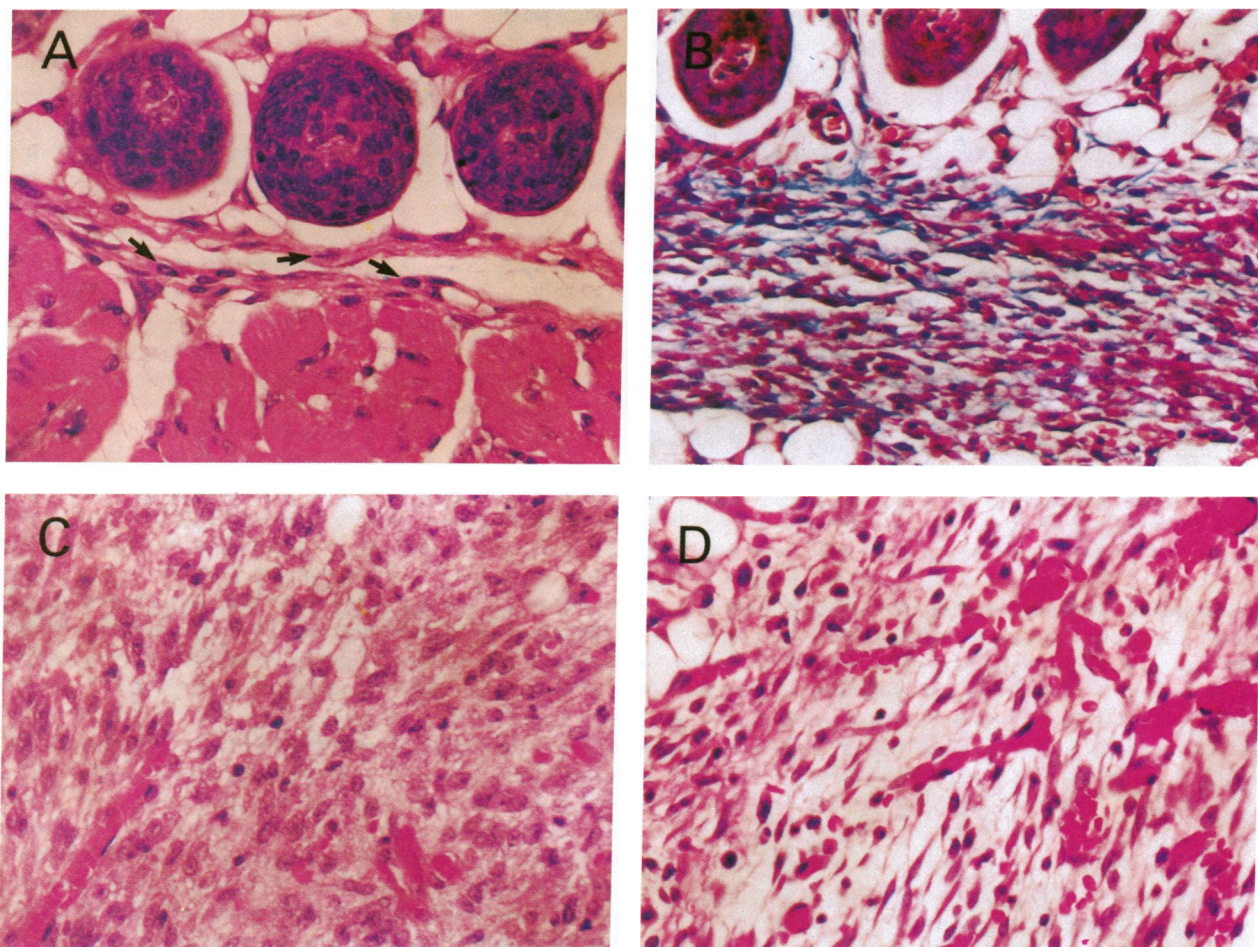


FIG. 1. Histologic effects of TGF- $\beta$  on induction of a fibrotic and angiogenic response after subcutaneous injection. Mice (1 day old) were injected each day in the nape of the neck with 20  $\mu$ l of a solution of saline alone or a saline solution of TGF- $\beta$  (800 ng); tissue samples were obtained as described in *Materials and Methods*. (A) Control injection (72 hr). This section shows the interface below the reticular dermis, between the subcutaneous adipose tissue (containing hair follicles, top), and the underlying skeletal muscle (bottom). Only a small number of fibroblasts are normally found at this interface, as indicated by arrows. (hematoxylin and eosin;  $\times 430$ .) (B) TGF- $\beta$  injection (48 hr). The subcutaneous interface is expanded by fibroblasts, macrophages, granulocytes, and newly formed collagen bundles (blue). (Masson trichrome;  $\times 260$ .) (C) TGF- $\beta$  injection (72 hr). The subcutaneous space is now further expanded by sheets of fibroblasts, endothelial cells, and macrophages, surrounded by a collagenous network. (hematoxylin and eosin;  $\times 430$ .) (D) TGF- $\beta$  injection (72 hr). This section shows pronounced neovascularization, with newly formed capillary loops, surrounded by fibroblasts and occasional macrophages. Extravasated erythrocytes are also present. (hematoxylin and eosin;  $\times 430$ .)

chase experiments (data not shown) have shown that the antagonistic effects of these two growth factors do not result

Table 1. Stimulation of proline incorporation into collagen by human dermal fibroblasts

Treatment	Incorporation of [ $^{14}$ C]proline into collagen, cpm per cell		Relative collagen biosynthesis, %	
	Donor 1	Donor 2	Donor 3	Donor 4
Control	1.5 $\pm$ 0.3	1.5 $\pm$ 0.7	7.7 $\pm$ 1.0	8.2 $\pm$ 1.0
TGF- $\beta$ (0.2 nM)	ND	ND	14.0 $\pm$ 0.4	13.0 $\pm$ 2.2
TGF- $\beta$ (0.4 nM)	12.0 $\pm$ 1.8	9.0 $\pm$ 1.6	19.0 $\pm$ 2.1	18.0 $\pm$ 1.9
EGF (0.8 nM)	1.4 $\pm$ 0.5	1.4 $\pm$ 0.5	ND	ND
EGF (0.8 nM) plus TGF- $\beta$ (0.4 nM)	2.3 $\pm$ 0.8	1.7 $\pm$ 0.8	ND	ND

Normal human dermal fibroblasts of four different donors were used in their third *in vitro* passage. Cells were treated with growth factors for 40 hr prior to labeling with [ $^{14}$ C]proline (2  $\mu$ Ci) for 20 hr in the presence of ascorbate (25  $\mu$ g/ml) and  $\beta$ -aminopropionitrile (20  $\mu$ g/ml). Determinations of secreted collagen were as described in *Materials and Methods*. Results are the mean  $\pm$  SD of triplicate determinations. ND, not determined.

from activation of an extracellular protease or collagenase activity by EGF. Incorporation of [ $^3$ H]proline into secreted collagen following a 2-hr pulse of cells treated with either 50 pM TGF- $\beta$  or 1 nM EGF (or 300 pM PDGF) remains stable for up to 4 hr after addition of unlabeled proline to a final concentration of 10 mM (chase).

Since some of the effects of growth factors on formation of new protein may be mediated by their ability to stimulate uptake of free amino acids into cells (20, 25–28), we have compared the ability of TGF- $\beta$ , EGF, and PDGF to stimulate uptake of [ $^{14}$ C]methylaminoisobutyric acid into NRK cells, as well as the ability of these three growth factors to stimulate [ $^3$ H]proline incorporation into collagen, as shown in Fig. 3. Methylaminoisobutyric acid, an amino acid that is not metabolized, was chosen because it is taken up by cells by system A transport (26), the same system that accounts for proline and glycine uptake. Although all three growth factors stimulated methylaminoisobutyric acid uptake, TGF- $\beta$  alone increased incorporation of proline into collagen; thus, the differential effects of these growth factors on proline incorporation into collagen apparently are not the result of effects on amino acid uptake. Confirmation of these results is seen in Fig. 4, which compares the effects of TGF- $\beta$ , PDGF, and EGF on incorporation of [ $^3$ H]leucine (which is taken up by

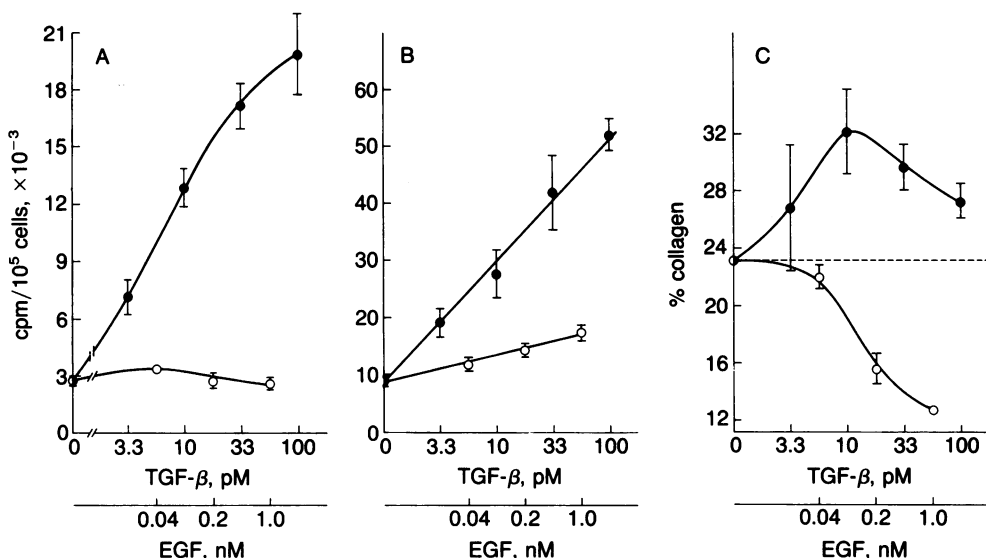


FIG. 2. Effects of TGF- $\beta$  (●) and EGF (○) on the incorporation of [ $^3$ H]proline into collagen and noncollagen proteins secreted into the medium of NRK cells. (A) Effects on incorporation into collagen; (B) effects on incorporation into noncollagen proteins; (C) effects on relative collagen biosynthesis. Experiments were carried out as described. Results are the mean  $\pm$  SD of triplicate determinations, each of which represents the pooled media of three wells. Incorporation was normalized to cell number, and percent collagen was calculated by an established formula (19).

cells by system L transport; ref. 26) into collagen, as well as on proline incorporation. It is clear that the use of leucine as a tracer gives the same relative results as seen with proline.

**TGF- $\beta$  in Media Conditioned by Lymphocytes.** Recently, activated lymphocytes have been shown to produce mRNA for TGF- $\beta$  (9) and to secrete TGF- $\beta$  itself (17); there is a 10- to 30-fold increase in TGF- $\beta$  in conditioned media of activated T lymphocytes compared to media obtained from unactivated lymphocytes (Fig. 5; ref. 17). To test whether TGF- $\beta$  secreted by lymphocytes might play an intrinsic role in formation of granulation tissue *in vivo*, we assayed the media conditioned by activated T lymphocytes for its ability to stimulate incorporation of proline into collagen. Fig. 5 shows that when lymphocytes are stimulated by phytohemagglutinin, there is a marked increase in both TGF- $\beta$  secretion and the ability of their conditioned medium to enhance the incorporation of [ $^3$ H]proline into collagen. Although activated lymphocytes produce many growth factors other than TGF- $\beta$  (12-16), experiments using rabbit anti-TGF- $\beta$  antibodies demonstrate that this peptide may be a significant mediator of the effects of lymphocytes on collagen formation in fibroblasts; preincubation in 2% plasma of anti-TGF- $\beta$  IgG (40  $\mu$ g per ml) with either standard platelet TGF- $\beta$  or tonsillar T-lymphocyte conditioned media (at final concentrations up to 50 pM TGF- $\beta$ ) blocked 89% of the

activity of platelet TGF- $\beta$  and 59% of the activity of the lymphocyte conditioned media, when compared to the stimulation obtained from samples treated with equal amounts of preimmune IgG.

## DISCUSSION

We have shown that TGF- $\beta$  has marked effects on the formation of collagen, both *in vivo* and *in vitro*, and that TGF- $\beta$  can also cause a striking angiogenic response in the newborn mouse. Furthermore, activation of lymphocytes causes them to produce amounts of TGF- $\beta$  that cause a marked stimulation of proline incorporation into collagen in model human and rodent *in vitro* systems. All of the above data, obtained from both *in vivo* and *in vitro* experiments, are compatible with the hypothesis that TGF- $\beta$  is a significant mediator of tissue repair.

The mechanism of action of TGF- $\beta$  in promoting angiogenesis is unknown and will involve an entire cascade of cellular and molecular events (30-34), including the possibilities that TGF- $\beta$  may be chemotactic for cells involved in angiogenesis, or even less directly that TGF- $\beta$  may induce cells to secrete other peptides with angiogenic activity. It has not yet been demonstrated that TGF- $\beta$  itself has any direct effect on proliferation of capillary endothelial cells.

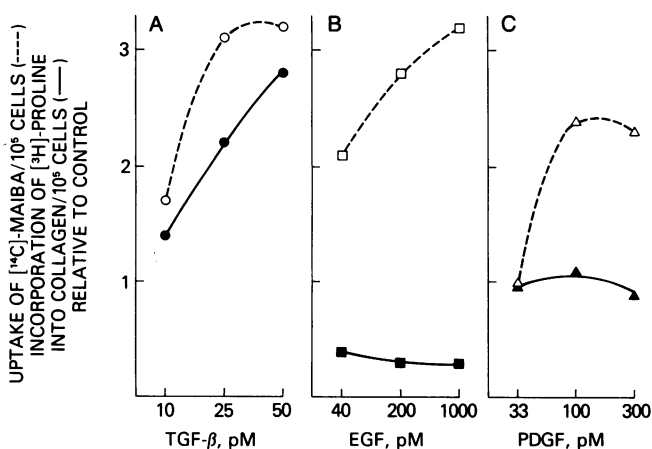


FIG. 3. Comparison of the effects of TGF- $\beta$  (A), EGF (B), and PDGF (C) on uptake of [ $^{14}$ C]methylaminoisobutyric acid (MAIBA) (open symbols) and incorporation of [ $^3$ H]proline into secreted collagen (solid symbols) in NRK cells. Incorporation into collagen was determined as described.

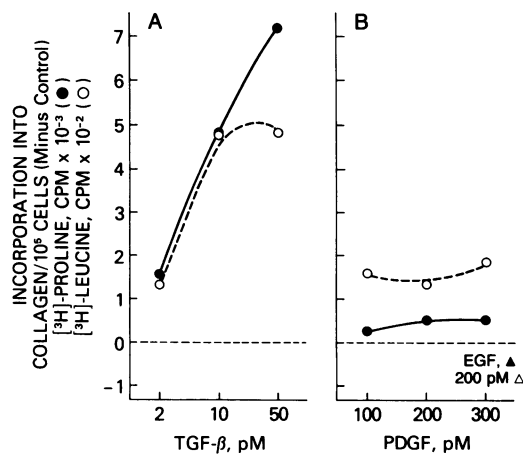


FIG. 4. Comparison of growth-factor-stimulated incorporation of proline (system A transport) and leucine (system L transport) into secreted collagen in NRK cells. Parallel incubations were carried out using either 6  $\mu$ Ci of [ $^3$ H]proline (solid symbols) or 12  $\mu$ Ci of L-[4,5- $^3$ H(N)]leucine (open symbols; 60 Ci/mmol) as described.

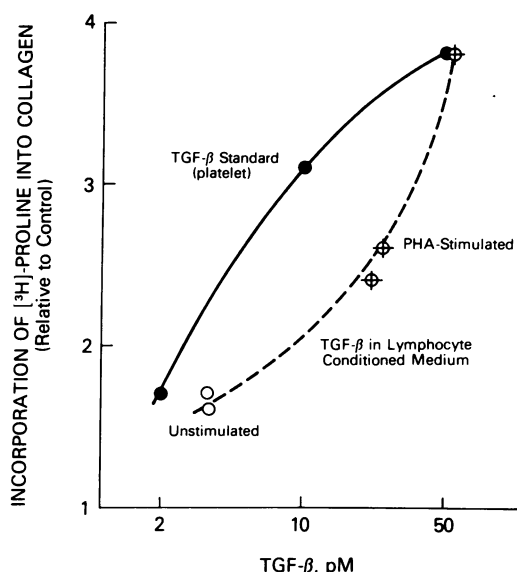


FIG. 5. Correlation of levels of TGF- $\beta$  (in medium conditioned by activated T lymphocytes) with stimulation of proline incorporation into collagen. Lymphocytes were cultured with or without phytohemagglutinin (PHA) (2  $\mu$ g/ml) for 4 days in serum-free (HB-102) medium (20 pM and 3.4 pM TGF- $\beta$ , respectively) or first for 2 days in serum-containing medium (RPMI 1640), then washed, and transferred to serum-free medium (HB 102) under the same conditions for an additional 4 days (50 pM TGF- $\beta$ ; ref. 17). Concentrations of TGF- $\beta$  in lymphocyte conditioned media (numbers in parentheses above) were determined both by assay of the soft agar colony-forming activity using NRK cells (1) and by a competitive radioreceptor binding assay using A549 cells (18) as described. Media were then assayed in the standard collagen assay using NRK cells, and the results were plotted against the TGF- $\beta$  concentrations determined above. As found previously for media conditioned by a variety of cells (29), the TGF- $\beta$  in media conditioned by lymphocytes was in an inactive latent form and required transient acid activation prior to assay.

The mechanism of action of TGF- $\beta$  in promoting collagen formation is similarly unknown. Certainly the increased numbers of fibroblasts at the site of injection (which might result from direct or indirect chemotactic actions of TGF- $\beta$  rather than direct proliferative actions) might be sufficient to explain the increased collagen deposition. However, *in vitro* experiments suggest a direct effect of TGF- $\beta$  on collagen synthesis. Preliminary experiments suggest that TGF- $\beta$  also increases formation of other matrix substances, such as glycosaminoglycans (V.F., unpublished results). In addition, although its functional implications are unknown, it may be noted that the TGF- $\beta$  precursor contains the minimal cellular recognition sequence, Arg-Gly-Asp-X, identified in the adhesion glycoprotein, fibronectin (35).

Finally, the known presence of TGF- $\beta$  in both platelets and activated lymphocytes suggests that this peptide may be an important intrinsic physiological paracrine mediator of repair. Although in this report we have emphasized the action of TGF- $\beta$  by itself, it is clear that *in vivo* TGF- $\beta$  acts in combination with other peptide growth regulators, such as EGF or its homolog, TGF- $\alpha$  (36), PDGF (36, 37), and basic fibroblastic growth factor (32), and that better understanding of its true role will require further study of these interactions.

We thank Russell Ross and Elaine Raines for a generous gift of pure PDGF and for helpful discussions, Beverly Peterkofsky for advice on methods for collagen, Gary Best and Edward Singletary for photomicrographs, and Sue Perdue for help with the manuscript.

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